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Functional Dissociation of μ Opioid Receptor Signaling and Endocytosis: Implications for the Biology of Opiate Tolerance and Addiction

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Summary

Opiate analgesia, tolerance, and addiction are mediated by drug-induced activation of the μ opioid receptor. A fundamental question in addiction biology is why exogenous opiate drugs have a high liability for inducing tolerance and addiction while native ligands do not. Studies indicate that highly addictive opiate drugs such as morphine are deficient in their ability to induce the desensitization and endocytosis of receptors. Here, we demonstrate that this regulatory mechanism reveals an independent functional property of opiate drugs that can be distinguished from previously established agonist properties. Moreover, this property correlates with agonist propensity to promote physiological tolerance, suggesting a fundamental revision of our understanding of the role of receptor endocytosis in the biology of opiate drug action and addiction.

Introduction

Alkaloid analgesic drugs such as morphine activate the same heptahelical receptors as native opioid peptide ligands. However, many opiate drugs are highly addictive, while endogenously released peptide ligands are not. An important distinction between opiate drugs and native peptides is that these two classes of ligand differ greatly in bioavailability and metabolism. Nevertheless, there are also significant differences in the ability of individual opiate drugs to induce physiological tolerance when administered at equieffective analgesic doses (Rezvani et al., 1983; Duttaroy and Yoburn, 1995; Mercadante et al., 1998). These results suggest that there may be significant variability in the actions of distinct alkaloid drugs on individual subtype(s) of opioid receptor, which could underlie differences in their liability for inducing the tolerance and dependence observed clinically and in animal studies. Importantly, targeted disruption of the μ opioid receptor gene abrogates analgesia, tolerance, and dependence induced by a variety of opiate drugs (Matthes et al., 1996), thus raising the possibility that differences in the addictive properties of individual opiate ligands reflect differences in the functional effects of these drugs on the μ opioid receptor itself.

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If this is the case, how could opiate drugs differ in their effects on the μ opioid receptor? It is well established that individual opiate drugs differ quantitatively in the affinity with which they bind receptors or the strength with which they activate receptors after binding. However, there is evidence that more fundamental qualitative differences between ligand effects may exist, particularly in the regulation of receptor signaling following activation. Specifically, individual opiate ligands differ greatly in their ability to induce rapid endocytosis of opioid receptors both in cultured cells and native neurons (Keith et al., 1996, 1998; Sternini et al., 1996). As this process is associated with functional desensitization of receptor-mediated signal transduction (Whistler and von Zastrow, 1998; Zhang et al., 1998), differential effects of opiate drugs on this regulatory mechanism may be of great physiological importance.

As intriguing as this possibility may be, it is also conceivable that differences in the abilities of individual opiate drugs to facilitate regulation of receptor-activated signaling by endocytosis may merely reflect previously recognized quantitative differences in the relative strength with which these individual drugs initially activate receptors and thus provide no additional insight into opiate drug action. Indeed, a close correlation between activation and endocytic regulatory actions of ligands for other heptahelical receptors has been reported (Szekeres et al., 1998), and a similar relationship has been suggested to exist for opiate drugs (Kovoor et al., 1998). Thus, the question of whether the differential effects of clinically important opiate drugs on the endocytic regulation of opioid receptors represents an independent functional property of opiate agonists is of critical importance to understanding the action of addictive opiate drugs. This question is especially significant in light of the well-established role of endocytosis in the desensitization and downregulation of receptor-mediated signaling, both of which have been implicated in the development of tolerance and dependence to opiate drugs. In addition, because heptahelical receptors comprise the largest class of neurotransmitter receptors and are the molecular targets of most neuropsychiatric drugs, this question is also of fundamental relevance to molecular neurobiology.

We have addressed this important question by directly comparing agonist activity of individual opiate ligands with their ability to induce regulatory endocytosis of μ opioid receptors in the same cell background. Using this approach, we demonstrate that the abilities of certain clinically important opiate drugs to activate receptor-mediated signaling and endocytosis are not directly correlated and are, in fact, inversely correlated in some cases. Furthermore, we identify a cytoplasmic domain of the μ opioid receptor that specifically controls the ability of opiate agonists to induce receptor endocytosis, thereby confirming mutationally the dissociation between these functional properties of individual ligands. Moreover, we demonstrate that altering the ability of certain opiate drugs to induce regulatory endocytosis of receptors causes profound effects on drug-induced

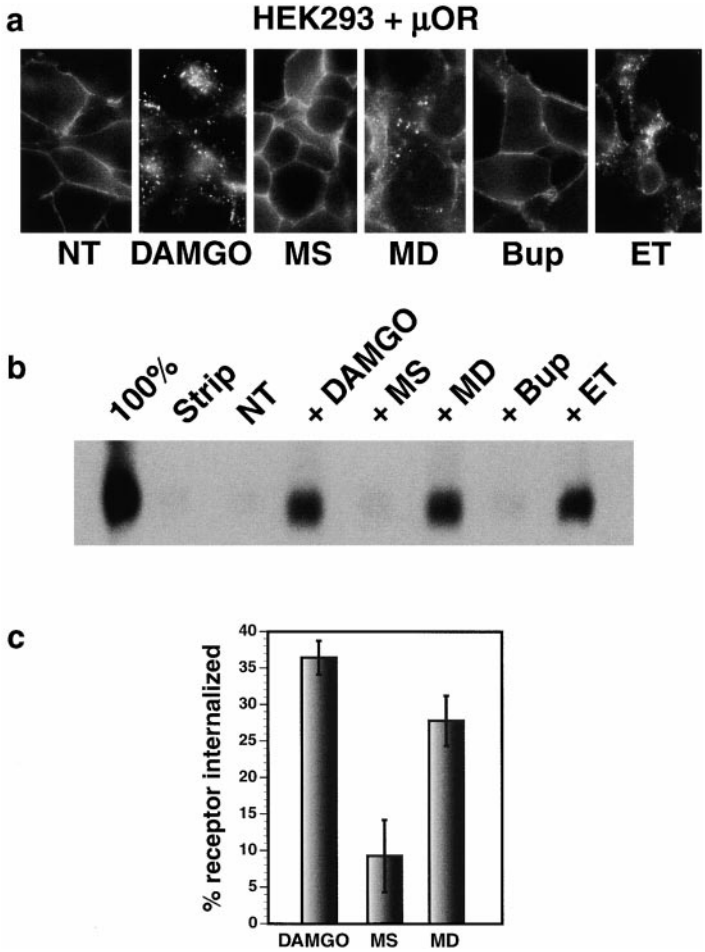

Figure 1. μ Opioid Receptors Demonstrated Agonist-Selective Endocytosis

(a) FLAG-tagged μ opioid receptors (μORs) remained predominantly in the plasma membrane in cells incubated in the absence of agonist (NT). μORs in cells incubated with 5 μM of the agonists DAMGO, methadone (MD), or etorphine (ET) were endocytosed, as indicated by redistribution of antibody-labeled receptors from the plasma membrane to numerous endocytic vesicles. Morphine (MS) or the mixed agonist/antagonist buprenorphine (Bup) failed to induce detectable endocytosis of the μOR.

(b) Internalization of receptors in response to each agonist examined in (a) was confirmed biochemically using cell surface biotinylation and protection, where internalized receptors are protected from cleavage by membrane-impermeant reducing agent.

(c) The relative activity of the agonists DAMGO, morphine (MS), and methadone (MD) for causing rapid internalization of the μOR was quantitated by immunofluorescence flow cytometry.

desensitization of receptor-mediated signal transduction. Finally, we demonstrate that mechanisms distinguishing the endocytic regulatory effects of opiate drugs are conserved when mutant receptors are expressed in primary-cultured neurons, suggesting that these observations are likely to be relevant to the physiological action of opiate drugs on native neurons.

Thus, we have defined a novel functional property that distinguishes individual opiate drugs and is distinct from previously defined parameters that characterize these drugs. These results constitute direct evidence that drugs can selectively manipulate distinct functional properties of any heptahelical receptor, and they suggest a significant revision to the well-accepted molecular mimicry hypothesis of opiate drug action. Furthermore, as the relative activity of certain opiate drugs to induce regulatory endocytosis of receptors appears to be inversely correlated with previous estimates of the relative ability of these drugs to induce physiological tolerance in vivo (Rezvani et al., 1983; Duttaroy and Yoburn, 1995; Mercadante et al., 1998), our findings suggest a fundamentally different hypothesis for the role of opioid receptor regulation in the biology of drug addiction.

Results

We examined ligand-induced signaling and endocytosis of an epitope-tagged μ opioid receptor expressed in stably transfected HEK 293 cells (~10^5 receptors/cell). Receptors expressed in this manner are functional and exhibit membrane trafficking properties closely similar to those of native receptors expressed endogenously in native neurons (Keith et al., 1996, 1998; Sternini et al., 1996), and rapid endocytosis of receptors observed in these cells is a reliable indicator of arrestin-dependent regulation of downstream signaling (Whistler and von Zastrow, 1998; Zhang et al., 1998).

While the opioid peptide DAMGO promoted the rapid endocytosis of μ opioid receptors, morphine failed to promote detectable endocytosis of receptors following prolonged activation, even when present at extremely high concentrations (Figure 1a). The failure of morphine to stimulate the rapid endocytosis of μ opioid receptors was not a general property of alkaloid agonists, as two other clinically relevant drugs structurally related to morphine, etorphine and methadone, stimulated rapid endocytosis of μ opioid receptors as efficiently as peptide agonist (Figure 1a). These observations were confirmed at saturating agonist concentration (to obviate any possible effects of agonist potency) using two independent quantitative assays for net receptor internalization (cell surface biotinylation and protection, Figure 1b, and fluorescence flow cytometry, Figure 1c). These experiments established the relative rank order of selected agonists for inducing endocytosis of opioid receptors as DAMGO ≈ methadone >>> morphine.

Opioid receptors signal by catalyzing ligand-dependent nucleotide exchange on G_i and G_o, thereby inhibiting adenylyl cyclase, inhibiting N-type calcium channels, and activating G protein–gated inwardly rectifying potassium (GIRK) -type potassium channels. Previously, we and others have demonstrated that morphine and DAMGO inhibit adenylyl cyclase to a similar extent in cells expressing μ opioid receptors at levels similar to or greater than those observed in native neurons (Traynor and Nahorski, 1995; Keith et al., 1996). However, opioid receptor–mediated inhibition of adenylyl cyclase activity in these cells typically does not require full receptor occupancy (Chavkin and Goldstein, 1984; Costa et al., 1992), and maximal inhibition of adenylyl cyclase observed in various cell types is independent of receptor density (Chakrabarti et al., 1995). Thus, this signaling readout is relatively insensitive to differences in agonist efficacy. In contrast, opioid receptor–induced activation of inwardly rectifying potassium channels requires significantly higher levels of agonist occupancy and is dependent on receptor density (Kovoor et al., 1998), thereby allowing quantitative differences in the agonist activity of individual ligands to be delineated in cells expressing physiologically relevant levels of receptor protein.

We introduced GIRK1/4 channels by stable transfection into the same μ opioid receptor–expressing cells in which the endocytosis assays were performed. To determine relative activities of individual agonists for inducing G protein–mediated signal transduction, we measured receptor-dependent activation of GIRK currents in whole cells. While the agonists tested were chosen because they exhibit similar potencies and affinities at the μ opioid receptor, all agonists were applied at a saturating concentration (1 μM) to obviate any possible effects of agonist potency on receptor-mediated signaling. To assure reliable within-cell comparison in all cases, whole-cell currents were recorded while morphine was applied first and then washed away, and the second agonist (morphine, DAMGO, or methadone) was applied immediately thereafter (Figure 2a). While morphine was observed to be a marginally less active agonist than DAMGO in some cells, there was no significant difference detected between the relative agonist activities of morphine and DAMGO using this assay when electrophysiological responses were averaged over multiple cells. In contrast, methadone was a substantially less-active agonist of GIRK activation in all cells examined, and a significant reduction in relative agonist activity (compared to both DAMGO and morphine) was confirmed by analysis of multiple cells (Figure 2b). Thus, the rank order of agonist activity for receptor-mediated signal transduction in these cells stably expressing μ opioid receptors and a GIRK1/4 channel was DAMGO ≥ morphine >> methadone (Figure 2b), consistent with the relative order of agonist activity measured in other systems (Yu et al., 1997) but in marked contrast to the rank order of these agonists to promote endocytosis of receptors (see Figure 1). These results indicate that morphine truly is different from other opiate ligands. Specifically, differential effects of the individual opiate drugs morphine and methadone on internalization of opioid receptors can be dissociated from quantitative

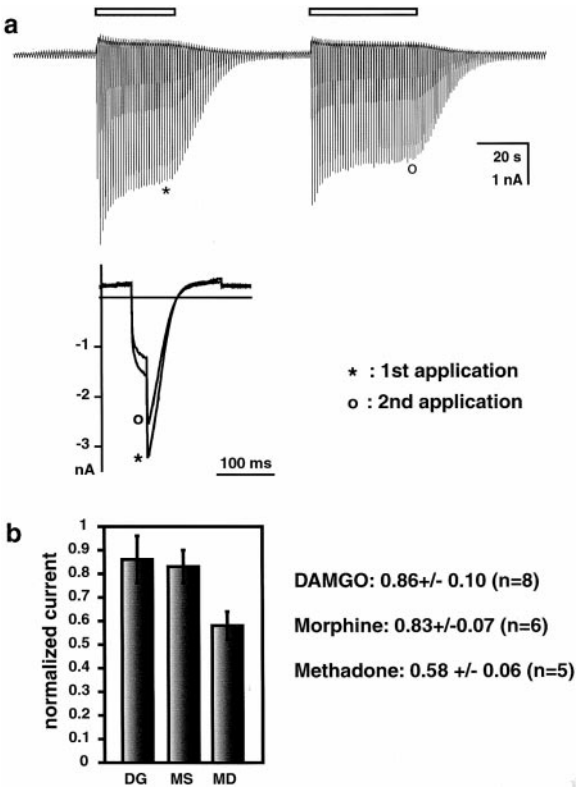

Figure 2. Relative Order of Activity of μ Opioid Receptor Ligands for Inducing G Protein–Mediated GIRK Activation

(a) The ability of saturating concentrations (1 μM) of morphine, DAMGO, and methadone to activate GIRK1/4 was measured in cells stably transfected with μORs and GIRK1/4 channels. To determine relative signaling activities, pair-wise agonist application was adopted. Receptors were activated first with 1 μM morphine, followed by washout and application of the second agonist also at 1 μM. Relative activity for each agonist was expressed as the ratio of the two steady-state currents (second agonist/first morphine). The continuous trace shown on top was from two pulses of 1 μM morphine application. The current–voltage relationship of the induced GIRK current was revealed by the ramp pulse. Membrane was held at 0 mV, followed by two 5 ms steps to +10 and +20 mV, and then jumped to 60 mV for 10 ms to assure correct activation kinetics of GIRK currents. Immediately after the three test pulses, membrane potential was ramped from 100 to +20 mV in a 200 ms interval.

(b) Slope conductances were measured in multiple cells between 90 and 70 mV to reflect the level of GIRK activation induced by ligand. By this measurement, morphine's ability to activate receptor was similar to that of DAMGO and significantly greater than that of methadone.

differences in the ability of these drugs to induce receptor-mediated signaling via heterotrimeric G proteins.

We next examined whether the differential effects of individual opiate drugs on receptor signaling and endocytosis could also be dissociated mutationally. In particular, we examined whether modification of cytoplasmic structures could influence the agonist selectivity of endocytosis. We replaced the cytoplasmic tail of the μ opioid receptor with the cytoplasmic tail of the δ opioid receptor. This chimeric receptor is a functional opioid receptor that exhibits agonist affinity similar to that of the wild-type μ opioid receptor (Afify et al., 1998). Remarkably, although neither the wild-type μ nor the δ

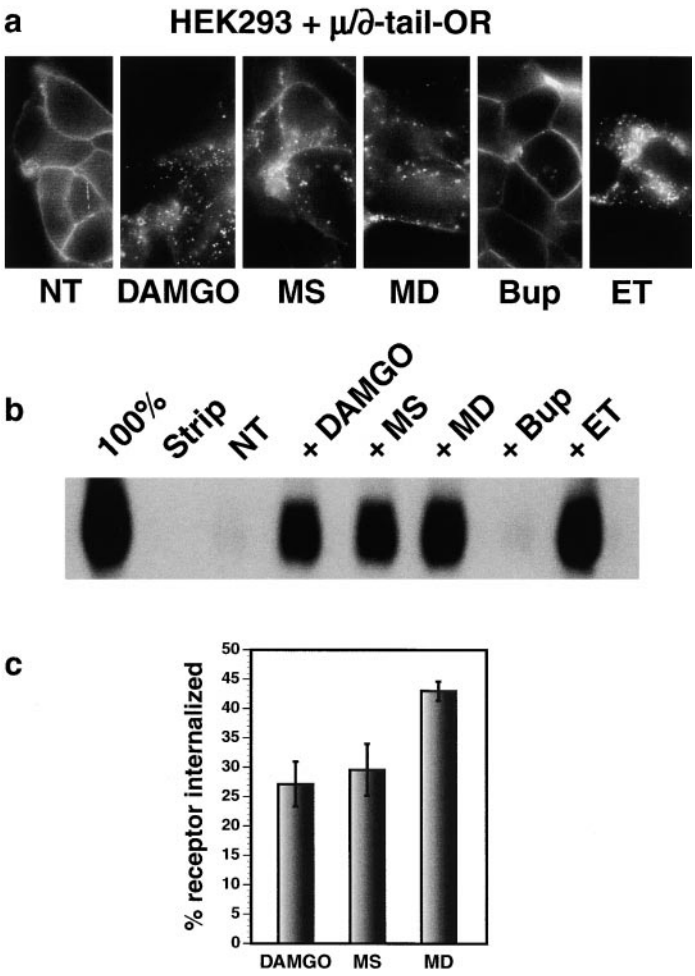

Figure 3. μ/δ-Tail Chimeric Receptors Demonstrated Altered Agonist Selectivity for Endocytosis

(a) FLAG-tagged μ/δ-tail chimeric receptors (μ/δ-tail-ORs) remained predominantly in the plasma membrane in cells incubated in the absence of agonist (NT). As for the μOR (Figure 1), μ/δ-tail-ORs internalized rapidly in the presence of 5 μM of the agonists DAMGO, methadone (MD), or etorphine (ET) but not buprenorphine (Bup). In contrast to the wild-type μOR, μ/δ-tail-ORs internalized rapidly in response to morphine (MS).

(b) Internalization of μ/δ-tail-ORs in response to each agonist examined in (a) was confirmed biochemically using cell surface biotinylation and protection.

(c) The relative activity of DAMGO, morphine (MS), and methadone (MD) for inducing rapid internalization of μ/δ-tail-ORs was quantitated by immunofluorescence flow cytometry.

opioid receptor endocytoses following activation by morphine, morphine-induced endocytosis of the chimeric receptor was readily observed (Figures 3a–3c). This enhanced endocytosis is unlikely to reflect an enhanced ability of the chimeric receptor to couple to heterotrimeric G proteins, because the ability of agonists to induce G protein–mediated inhibition of adenylyl cyclase via this receptor is not enhanced (and is, in fact, slightly reduced) relative to the wild-type μ opioid receptor (Afify et al., 1998). Thus, mutation of a single cytoplasmic domain appears to specifically influence the agonist selectivity of opioid receptor endocytosis.

We next investigated whether altering the cytoplasmic tail of the μ opioid receptor altered the relative order of activity of morphine and DAMGO for inducing G protein–mediated signal transduction. HEK 293 cells expressing a GIRK1/2 channel (Chuang et al., 1998) were transiently transfected with either the μ or the chimeric opioid receptor. This experimental design allowed agonist activity to be examined in cells that express receptors over a range of expression levels. Consistent with our results in Figure 2, which measured GIRK1/4 activation in stably transfected cells uniformly expressing the μ opioid receptor, the agonist activity of morphine was closely similar to that of DAMGO in many of the transiently transfected cells (Figure 4a). However, in some cells, those we assume from previous studies (Kovoor et al., 1998) to express much lower levels of receptor protein, the relative difference between morphine and DAMGO was more pronounced (compare Figures 4a and 4b).

Essentially identical results were obtained when the relative agonist activities of morphine and DAMGO were compared in cells transiently transfected with the chimeric receptor (Figures 4c and 4d). The relative order of activity for DAMGO and morphine for both the μ and the chimeric receptor was confirmed by measuring ligand-induced GIRK currents in multiple cells selected at random from each cell population. This analysis indicated that the tail substitution caused no detectable effect on the relative agonist activity of morphine at the chimeric receptor (Figure 4e). Importantly, we observed that the relative activity of morphine and DAMGO for both μ and chimeric receptors was independent of the order in which the agonists were applied. These observations confirm that the mutation of the receptor tail specifically affected the relative activity of morphine for inducing receptor endocytosis without changing the relative agonist activity for G protein–mediated signaling, suggesting that the carboxy-terminal cytoplasmic domain of the receptor protein is able to distinguish the regulatory properties of distinct activated conformations of the receptor protein induced by structurally distinct agonists.

The chimeric opioid receptor provided an opportunity

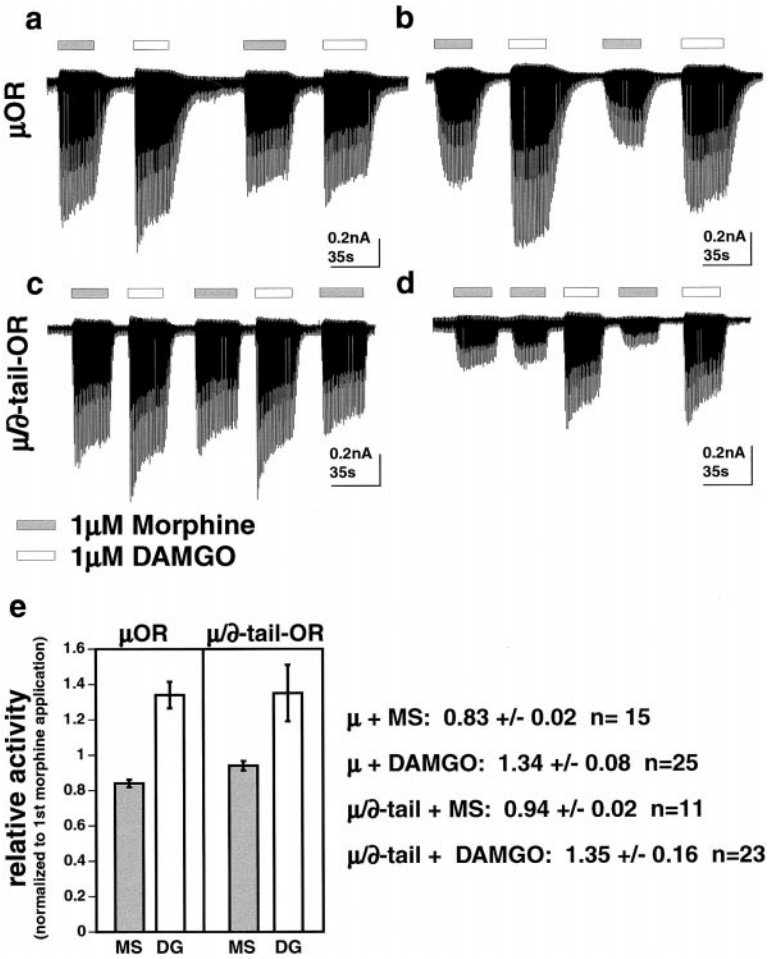

Figure 4. The Relative Order of Activity of Morphine and DAMGO Was Not Altered by Replacing the Cytoplasmic Tail of the Receptor

HEK 293 cells expressing a GIRK1/2 channel (Chuang et al., 1998) were transiently transfected with either the μ opioid receptor (a and b) or the chimeric receptor (c and d). Agonist was applied and recordings made as for Figure 2. Receptor-mediated activation of GIRK currents by morphine and DAMGO were similar in many cells examined in the populations of μ (a) and chimera (c) -expressing cells, consistent with the results obtained in Figure 2. In some cells present in the transiently transfected populations expressing either μORs or μ/δ-tail-ORs at relatively low levels (see Discussion in text), differences between the signaling activities of morphine and DAMGO were more pronounced (b and d). In (e), quantitation of these data over a large number of cells, chosen at random from the transiently transfected populations, revealed no significant difference in the relative signaling activity of morphine and DAMGO at the μ versus the chimeric receptor.

to examine the functional consequences of altering agonist-specific endocytosis. To avoid complications that may arise from desensitization due to the G protein cycle or effector regulation upon agonist treatment (Chuang et al., 1998), we examined directly the coupling between μ and chimeric opioid receptor proteins and G protein by measuring nucleotide exchange activity in isolated membranes. μ opioid receptors rapidly uncoupled from heterotrimeric G proteins following activation by the peptide agonist DAMGO but not following activation by morphine (Figure 5a). In contrast, the chimeric receptor exhibited nearly complete uncoupling from G protein following activation by both DAMGO and morphine (Figure 5b). These results confirm that the receptor mutation changes the agonist selectivity of not only endocytosis but also receptor–G protein uncoupling, both of which influence downstream signaling (Whistler and von Zastrow, 1998).

It is somewhat remarkable that, while neither the μ nor the δ opioid receptor endocytoses following activation by morphine (Keith et al., 1996), a chimeric receptor derived from these receptors does so with high efficiency. The wild-type δ opioid receptor binds morphine poorly compared to the μ receptor (Raynor et al., 1994). In contrast, the chimeric mutant receptor has similar pharmacology to the μ receptor (Afify et al., 1998), consistent with the transmembrane and extracellular domains involved in ligand recognition being identical to the μ receptor. Therefore, we examined the mechanism by which the alteration of a single cytoplasmic domain, which is not expected to influence the structure of the heptahelical receptor "core" critical for ligand recognition and agonist-induced conformation change, promoted morphine-induced endocytosis of the chimeric receptor.

Both the μ opioid receptor and the δ opioid receptor endocytose via a dynamin-dependent mechanism involving clathrin-coated pits (Keith et al., 1996; Whistler and von Zastrow, 1998). We observed that endocytosis of the chimeric receptor, in response to both DAMGO and morphine, was also mediated by a dynamin-dependent mechanism (data not shown). Previously, we and others have demonstrated that overexpression of either arrestin (Whistler and von Zastrow, 1998) or G protein–coupled receptor kinase (GRK) (Zhang et al., 1998) can facilitate endocytosis of morphine-activated opioid receptors, suggesting that morphine-activated receptors are in a conformation that is recalcitrant to the GRK-mediated phosphorylation and arrestin binding involved in promoting dynamin-dependent endocytosis. Indeed, extensive previous studies indicate that both δ and μ opioid receptors are phosphorylated on multiple serine/threonine sites located in the carboxy-terminal cytoplasmic tail (Pei et al., 1995; Trapaidze et al., 1996; Zhang et al., 1996). Therefore, we investigated the hypothesis that the tail substitution generates a chimeric receptor

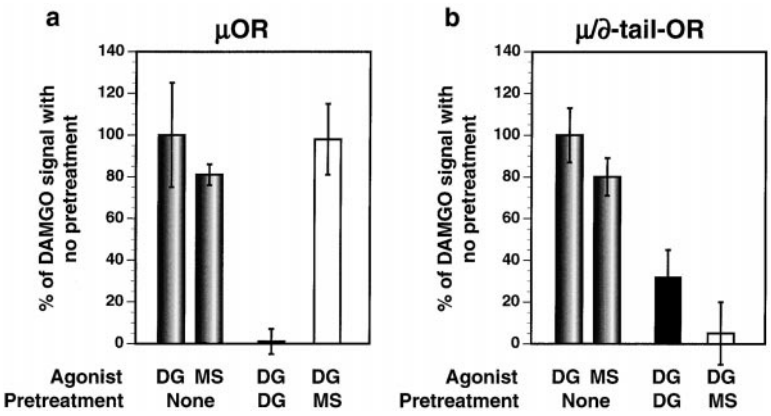

Figure 5. μ/δ-Tail Chimeric Receptors Exhibited Functional Uncoupling from G Protein following Morphine Activation

Cells stably expressing either μORs or μ/δ-tail-ORs were pretreated for 5 min with DAMGO (DG) or morphine (MS) or left untreated, and receptor–G protein coupling was estimated by assaying the GTPγS binding induced by agonist rechallenge of washed membranes. Receptors from both cell lines that were not pretreated with agonist stimulated GTP exchange efficiently with both DAMGO (DG) and morphine (MS) ([a] and [b], shaded bars). μORs pretreated with DAMGO (DG) were very inefficient at promoting GTP exchange upon DAMGO restimulation ([a], closed bar), indicating that the μORs in these cells had become uncoupled from G protein during the DAMGO pretreatment. μORs pretreated with morphine (MS) were still as effective as untreated receptors at promoting GTP exchange upon DAMGO restimulation ([a], open bar) indicating that pretreatment with morphine failed to uncouple the receptors from G protein. μ/δ-tail-ORs pretreated with DAMGO (DG) were also inefficient at promoting GTP exchange upon DAMGO restimulation ([b], closed bar), indicating that the chimeric receptors in these cells had become uncoupled from G protein during the DAMGO pretreatment. In addition, μ/δ-tail-ORs pretreated with morphine (MS) were also very ineffective at promoting GTP exchange upon DAMGO restimulation ([b], open bar), differing markedly from morphine-pretreated μORs and demonstrating that morphine facilitated functional uncoupling of the chimeric receptor from G protein.

that is a better substrate for regulatory phosphorylation and arrestin interaction than is the wild-type μ opioid receptor when these receptors are in the morphine-activated conformation.

The wild-type μ opioid receptor was phosphorylated in intact HEK 293 cells at a low level in the absence of agonist, and receptor phosphorylation was enhanced ~2-fold when receptors were activated with etorphine (Figure 6a) or DAMGO (data not shown). Consistent with our results and previous studies of μ receptor phosphorylation in various cell types (Zhang et al., 1996, 1998), morphine failed to cause any detectable phosphorylation of the wild-type μ opioid receptor above the constitutive background level (Figure 6a). Wild-type δ receptors expressed at similar levels are strongly phosphorylated following activation by opioid peptide or etorphine (Murray et al., 1998) but not morphine (data not shown), consistent with previous studies demonstrating that the cytoplasmic tail of the δ opioid receptor is a highly favorable site for GRK-mediated phosphorylation (Pei et al., 1995) and that this receptor exhibits more pronounced GRK-mediated desensitization than the μ receptor in *Xenopus* oocytes (Kovoor et al., 1997). Phosphorylation of the chimeric receptor, like the wild-type δ receptor, was strongly stimulated by etorphine (Figure 6a) or DAMGO (data not shown). Remarkably, phosphorylation of the chimeric receptor was also strongly stimulated by morphine (Figure 6a), in marked contrast to the absence of detectable morphine-induced phosphorylation of the wild-type μ or δ opioid receptor.

We next examined whether alterations in ligand-dependent phosphorylation were associated with differences in membrane recruitment of β-arrestins. Consistent with its failure to induce detectable phosphorylation or rapid endocytosis of the wild-type μ opioid receptor, saturating concentrations of morphine failed to cause detectable membrane recruitment of GFP-tagged arrestin 3 (β-arrestin 2) (Figure 6b; see also Zhang et al., 1998) in cells expressing wild-type μ opioid receptor. In contrast, etorphine-activated μ receptors efficiently recruited GFP-tagged arrestin to membrane puncta (Figure 6b), in agreement with previous studies establishing the utility of GFP-tagged β-arrestin for monitoring agonist-specific regulation of opioid receptors (Barak et al., 1997; Zhang et al., 1998). Importantly, morphine-activated chimeric receptors strongly promoted recruitment of GFP–arrestin (Figure 6b), consistent with the pronounced morphine-induced phosphorylation of the chimera (Figure 6a). Taken together, these observations strongly support the hypothesis that we have created a gain-of-function phenotype in the chimeric receptor by combining the pharmacology of the μ opioid receptor with the enhanced kinase substrate activity of the δ tail. Thus, the chimeric receptor, in contrast to wild-type opioid receptors, can undergo sufficient morphine-induced phosphorylation to efficiently recruit β-arrestin and undergo rapid, dynamin-dependent endocytosis.

To examine whether conformational mechanisms mediating the agonist specificity of opioid receptor regulation in our transfected cell model system are physiologically relevant to native neurons, we utilized adenovirus-mediated gene transfer to express epitope-tagged versions of the wild-type or chimeric μ opioid receptor in primary cultures of rat hippocampal neurons. Both the wild-type and chimeric receptors were localized primarily in the plasma membrane of the cell body and neurites in the absence of agonist and, as expected, exhibited rapid internalization in the presence of DAMGO or etorphine (data not shown). However, the distribution of the two receptors following morphine stimulation was dramatically different. Wild-type μ opioid receptors remained in the plasma membrane following activation with morphine (Figure 7, left), consistent with the failure of morphine to induce endocytosis of native μ opioid receptors in neurons. In marked contrast, the chimeric receptor rapidly endocytosed in the presence of morphine when examined at similar expression levels under identical experimental conditions (Figure 7, right). Thus, mechanisms and receptor determinants mediating the ligand-selective endocytosis of opioid receptors are conserved, suggesting our observations are likely to be relevant to regulation of receptor-mediated signaling and thus the actions of opiate drugs in native neurons.

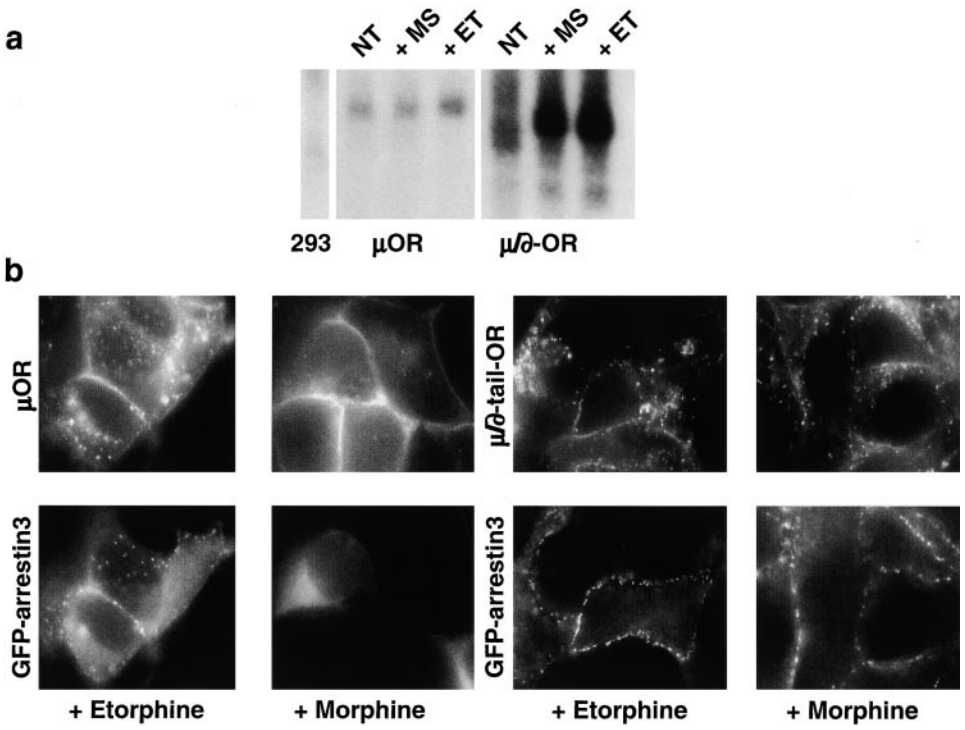

Figure 6. The μ/δ-Tail Chimeric Receptor Was Phosphorylated and Recruited Arrestin following Activation by Morphine

(a) HEK 293 cells stably expressing either FLAG-tagged μORs or μ/δ-tail-ORs were metabolically labeled with ^{32}P inorganic phosphate and treated with morphine, etorphine, or left untreated, and receptor phosphorylation was examined by SDS–PAGE autoradiography of anti-FLAG immunoprecipitates. Negligible nonspecific signal was observed in control immunoprecipitates from untransfected cells (293). In cells expressing μOR, morphine (MS) failed to induce detectable receptor phosphorylation above the constitutive level observed in cells incubated in the absence of agonist (NT). Incubation of cells with etorphine (ET) enhanced phosphorylation of the μOR ~2-fold. Phosphorylation of the μ/δ-tail-OR was much more strongly stimulated by etorphine (compare ET with NT in the right panel versus the left panel). In addition, morphine (MS) strongly stimulated phosphorylation of the μ/δ-tail-OR, in contrast to its lack of detectable effect on the μOR.

(b) HEK 293 cells stably expressing either μOR or μ/δ-tail-OR were transiently transfected with GFP–arrestin 3 (Barak et al., 1997) and incubated for 10 min in the presence of the indicated agonist, and localization of receptor and arrestin were examined by fluorescence microscopy. μOR (left) exhibited pronounced internalization in response to etorphine but not morphine (top). The corresponding images of GFP–arrestin (bottom) demonstrated that etorphine activation induced punctate membrane recruitment of arrestin, whereas activation of the μOR with morphine failed to cause membrane recruitment of arrestin. In contrast, the μ/δ-tail-OR (right) internalized (top) and mediated pronounced membrane recruitment of arrestin (bottom) following activation by both etorphine and morphine.

Discussion

Taken together, our results suggest that differences in the ability of select opioid agonists to mediate regulation of receptor signaling by rapid endocytosis represents an independent functional property that distinguishes clinically important opiate analgesic drugs such as morphine and methadone. This agonist property profoundly effects the regulation of downstream signaling and can be distinguished both pharmacologically and mutationally from other important functional parameters such as potency and intrinsic activity for receptor activation.

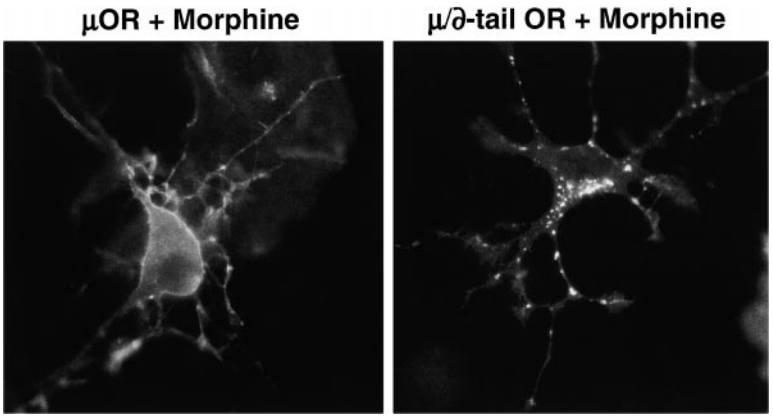

Figure 7. The μ/δ-Tail Chimeric Receptor Exhibited Morphine-Induced Endocytosis in Neurons

Rat hippocampal neurons from 3-week-old cultures were infected with adenovirus expressing epitope-tagged μOR or μ/δ-tail-OR and allowed to recover for 2 days. Cells were fed antibody to label cell surface receptors and then treated with 5 μM morphine, fixed, and processed for immunocytochemical localization of μOR (left) or μ/δ-tail-OR (right). While the μORs remained predominantly on the plasma membrane upon morphine stimulation, μ/δ-tail-ORs were rapidly redistributed to endocytic vesicles following activation by morphine.

Table 1. RAVE Values for Individual μ Opioid Receptor Agonists

	DAMGO	Morphine	Methadone
Activity (normalized current)	0.86	0.83	0.58
Relative activity (A)	1	0.97	0.67
Endocytosis (% receptor internalized)	37	9	28
Relative endocytosis (E)	1	0.25	0.78
RAVE (A/E)	1	3.88	0.86

RAVE value was defined as relative activity versus endocytosis or A/E. Activity in this case was measured as normalized current and endocytosis as percent receptor internalized measured by fluorescence flow cytometry. The peptide DAMGO was defined as having activity and endocytosis of 1 and thus a RAVE of 1. Morphine has a RAVE significantly greater than 1, reflecting its relatively poor ability to stimulate endocytosis despite its ability to activate signaling. Methadone has a RAVE somewhat less than 1, reflecting its relatively good ability to stimulate endocytosis despite its relatively poor ability to activate signaling.

Thus, the differences in endocytic regulation reflect fundamental differences between individual opiate drugs in their ability to facilitate the dynamic regulation of opioid receptor signaling. Importantly, while the existence of this distinct functional property has been established most clearly using a model cell system, our studies of primary cultured neurons suggest that the mechanisms underlying the agonist specificity for opioid receptor endocytosis also operate in native neurons, consistent with the pronounced agonist specificity of receptor internalization observed previously in studies of native μ opioid receptors in the intact brain (Keith et al., 1998).

One way to define this independent property of an individual ligand is to express its ability to activate receptors and drive their endocytosis as a ratio. Such a ratio can be derived from the present data using the relative ability of individual agonists to activate GIRK-type potassium channel conductance and promote rapid endocytosis of receptors in the same cell background. We refer to this independent property as RAVE (for relative activity versus endocytosis). If we define the peptide agonist DAMGO as having a RAVE of 1 (Table 1), then morphine would have a RAVE substantially greater than 1 (~4), as its relative ability to signal is much greater than its relative ability to stimulate receptor endocytosis. Conversely, methadone would have a RAVE somewhat less than 1 (~0.8) and closer to that of opioid peptide (1), reflecting the ability of methadone to induce regulatory endocytosis of receptors to a somewhat greater extent than its relative activity for promoting receptor-mediated signal transduction.

While use of this terminology emphasizes this functional difference between individual opiate agonists on G protein–mediated signaling and desensitization by endocytosis, this difference is unlikely to represent the only determinant of the differential effects of individual opiate drugs on receptor regulation. Indeed, opiate drugs with similar RAVE values would be expected to differ in their effects on receptor regulation by endocytosis directly in proportion to their relative efficacies for receptor activation as suggested in a recent study comparing several opiate agonists on μ opioid receptors expressed in *Xenopus* oocytes (Kovoor et al., 1998). Nevertheless, agonists with similar intrinsic activities could have different RAVE values if they promoted different levels of receptor endocytosis. For example, even under receptor-limited conditions in *Xenopus* oocytes, which enhance observed differences in relative agonist activity, the opiate agonists DAMGO and fentanyl have essentially identical intrinsic activities for GIRK activation (Kovoor et al., 1998). However, fentanyl stimulates less endocytosis in mammalian cells than does DAMGO (Keith et al., 1998). Thus, we would predict that fentanyl would have a RAVE value greater than that of DAMGO and more similar to that of morphine.

What might be the physiological consequences of these fundamental differences among individual drugs in their ability to induce the rapid regulation of opioid receptors by endocytosis? Clinical observations and animal studies indicate that significant differences do indeed exist among individual opiate analgesic drugs with respect to their tendency to promote physiological tolerance and dependence. For example, both methadone and etorphine have been reported to induce less tolerance than morphine (Rezvani et al., 1983; Duttaroy and Yoburn, 1995; Mercadante et al., 1998) when administered chronically at equieffective analgesic doses. Our estimates indicate that both of these drugs have RAVE values similar to those of opioid peptide and much less than morphine (Table 1 and data not shown). It will be important in future studies to examine the relationship between RAVE values of additional opiate drugs measured in cultured cells with their relative addictive liabilities observed in an animal model.

We propose that the arrestin-mediated regulation of opioid receptors by endocytosis (Whistler and von Zastrow, 1998; Zhang et al., 1998) may actually serve a *protective* role in reducing the development of physiological drug tolerance. Specifically, we propose that a suite of highly conserved receptor regulatory mechanisms, mediated by GRK and arrestin interaction with activated receptors (reviewed by Ferguson et al., 1998; Krupnick and Benovic, 1998; Lefkowitz et al., 1998), serves to rapidly attenuate receptor-mediated signaling. This regulation serves at least two roles. First, by functional uncoupling of receptor and G protein, followed by endocytosis, this arrestin-mediated regulation rapidly desensitizes cells to agonist. Second, following endocytosis, receptors can be recycled to the cell surface in a fully active state, thereby resensitizing cells to agonist. This dynamic cycle of receptor regulation may be designed to mediate the dynamic actions of native opioid peptides, which are typically released in a phasic or pulsatile manner.

Opiate drugs, in contrast, persist in the extracellular milieu for a prolonged period of time because of their resistance to proteolytic degradation and, hence, activate opioid receptors in an abnormally prolonged manner. Accordingly, opiate drugs that induce the rapid desensitization and endocytosis of receptors followed by resensitization to agonist may more closely mimic the phasic actions and physiological adaptations observed with native peptide ligands. In contrast, opiate drugs such as morphine persistently activate receptors, forcing other cellular mechanisms to compensate at downstream site(s) for this prolonged activation. Thus, morphine could have enhanced propensity to cause widespread changes in

neural plasticity associated with drug addiction precisely because of its failure to effectively promote efficient arrestin-mediated regulation of the μ opioid receptor itself.

In contrast to the prevailing hypothesis, which proposes that desensitization of opioid receptors directly contributes to physiological tolerance, our observations suggest that the *failure* of morphine-activated receptors to uncouple from G protein and endocytose appropriately may be critical for the high level of physiological tolerance induced by morphine. While the previous hypothesis was proposed based on the observation that morphine-tolerant animals exhibit a right-shifted dose–response curve for receptor-mediated signal transduction measured in tissue preparations, consistent with a reduction in receptor reserve (Chavkin and Goldstein, 1984), such a reduction in agonist potency observed over a prolonged time period is also consistent with other modifications of the signal transduction system. Indeed, a number of studies indicate that morphine fails to promote significant downregulation of μ opioid receptors even under conditions that induce profound physiological tolerance (Lenoir et al., 1984; Simantov et al., 1984). Thus, we anticipate that downstream regulatory responses induced by the failure of morphine to promote efficient arrestin-mediated desensitization may include additional modification(s) of the receptor itself that change the apparent functional receptor reserve independent from changes in total receptor number.

In conclusion, we have demonstrated that individual opiate drugs differ significantly in their effects on the rapid regulation of opioid receptors, and that these differences can be dissociated both pharmacologically and mutationally from differences among individual agonists in their ability to bind to and activate receptors. Moreover, mechanisms that underlie agonist-specific differences in opioid receptor regulation by endocytosis operate in native neurons. Thus, we have defined a functional property of opiate ligands that may be of fundamental importance to neural signal transduction and that suggests a significant revision of our understanding of the biological mechanisms underlying drug tolerance and addiction.

Experimental Procedures

Cell Culture and Immunocytochemistry

HEK 293 cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum (UCSF Cell Culture Facility). Cells were stained for receptor as previously described (Whistler and von Zastrow, 1998). Hippocampal cultures were prepared and maintained in neurobasal media with 4% B27 as previously described (Lissin et al., 1998). Three-week-old cultures were infected with a replication-defective adenovirus encoding the FLAG-tagged murine μ or chimeric opioid receptor (gift of Dr. Stephen Hardy) for 45 min, washed three times, and cultured for 48 hr prior to ligand application and immunocytochemical staining.

Assay of Receptor Internalization by Surface Biotinylation

Cells were grown to 80% confluency, incubated with biotin, and treated as previously described (Whistler and von Zastrow, 1998).

Immunofluorescence Flow Cytometry

Cells were treated with agonist for 30 min and stained and detected as previously described (Keith et al., 1996).

Guanine Nucleotide Exchange Assays

Membranes were prepared as previously described (Whistler and von Zastrow, 1998) after 5 min agonist treatment with either morphine or DAMGO. Methods were used as described previously for GTPγS binding (Whistler and von Zastrow, 1998) using membranes prepared as above unstimulated or stimulated with 5 μM DAMGO or morphine for 30 min at 25°C. Reactions were terminated by vacuum filtration.

Electrophysiology

In Stable Cell Line

HEK 293 cells stably expressing GIRK1/4 channels and μ opioid receptor were trypsinized 24–48 hr before experiments and plated onto coverslips. Experiments were performed in the tight-seal whole-cell mode. The bath solution contained 10 mM HEPES (pH 7.4), 20 mM KCl, 120 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 μM NaOH. The composition of the pipette solution was 9 mM HEPES (pH 7.2), 9 mM NaCl, 4.5 mM EGTA, 4.5 mM K_2HPO_4, 99 mM KCl, 1.8 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 mM Na_2GTP, and 18 mM NaOH. Series resistances of electrodes were within 3 MΩ and compensated to at least 95%. To measure the μ receptor–activated GIRK currents, a ramp protocol, as depicted in the Figure 2 legend, was applied repetitively every second. Whole-cell potassium currents were expressed as the total potassium conductance in the membrane. Data were filtered at 2 kHz and sampled at 1 kHz.

In Transiently Transfected Cells

HEK 293 cells expressing a GIRK1/2 channel (Chuang et al., 1998) were transiently transfected with either the μ or the chimeric opioid receptor and recordings were taken as above.

Receptor Phosphorylation and Immunoprecipitation

HEK 293 cells stably expressing either the FLAG-tagged μ or chimeric receptor were grown in DMEM + 10% fetal bovine serum to 80% confluency in 10 cm dishes and then starved for 1 hr in phosphate-free, serum-free media. Cells were metabolically labeled for 3 hr with ^{32}P orthophosphate (Amersham Life Sciences, 0.25 mCi/ml) and then incubated in the presence or absence of agonist for 30 min. Cells were washed two times with ice-cold PBS and lysed in 1 ml/dish extraction buffer (25 mM HEPES [pH 7.4], 50 mM NaCl, 0.1% Triton X-100, 1 mM $CaCl_2$, 50 mM NaF, 80 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 2 μg/ml phenylmethylsulfonyl fluoride), and receptors were immunoprecipitated from extracts using M1 anti-FLAG monoclonal antibody (Kodak Scientific Imaging Systems) and protein A-Sepharose (Amersham Pharmacia Biotech) at 4°C for 2 hr. Immunoprecipitates were washed extensively in lysis buffer without phosphatase and protease inhibitors, treated with PNGase F (New England Biolabs) in 10 mM Tris (pH 7.4) for 2 hr at 37°C and were resolved by SDS–PAGE.

Arrestin Recruitment

HEK 293 cells stably expressing μ or chimeric receptor were transiently transfected with GFP–arrestin (Barak et al., 1997), and allowed to recover for 24 hr. Cells were then plated to coverslips and allowed to grow for 24 hr. Cells were fed M1 anti-FLAG antibodies (Kodak Scientific Imaging Systems) for 30 min (1:1000, 3 μg/ml) to label receptors in the plasma membrane and then treated with agonist for 10 min. Cells were fixed and permeabilized as previously described (Whistler and von Zastrow, 1998) and then stained for receptor with Texas red–conjugated donkey anti-mouse secondary antibody (1:500, Jackson Immunoresearch). GFP-tagged arrestin and antibody-labeled receptor were visualized by conventional epifluorescence microscopy using a Nikon inverted microscope equipped with a 60× NA1.4 objective and standard filter sets (Omega Optical) allowing specific visualization of both fluorophores without detectable bleedthrough.

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